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Vegt Activation at the Midblastula Transition Alters the Response to Nodal Signals in the Vegetal Endoderm Domain

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In *Xenopus*, the prospective endoderm and mesoderm are localized to discrete, adjacent domains at the beginning of gastrulation, and this is made evident by the expression of *Sox17* in vegetal blastomeres and *Brachyury* (*Xbra*) in marginal blastomeres. Here, we examine the regulation of *Sox17α* expression and the role of Sox17α in establishing the vegetal endodermal gene expression domain. Injection of specific inhibitors of VegT or Nodal resulted in a loss of *Sox17α* expression in the gastrula. However, the onset of *Sox17α* expression at the midblastula transition was dependent on VegT, but not on Nodal function, indicating that *Sox17α* expression is initiated by VegT and then maintained by Nodal signals. Consistent with these results, VegT, but not *Xenopus* Nodal-related-1 (Xnr1), can activate *Sox17α* expression at the midblastula stage in animal explants. In addition, VegT activates *Sox17α* in the presence of cycloheximide or a Nodal antagonist, suggesting that *Sox17α* is an immediate-early target of VegT in vegetal blastomeres. Given that Nodal signals are necessary and sufficient for both mesodermal and endodermal gene expression, we propose that VegT activation of *Sox17α* at the midblastula transition prevents mesodermal gene expression in response to Nodal signals, thus establishing the vegetal endodermal gene expression domain. Supporting this idea, Sox17α misexpression in the marginal zone inhibits the expression of multiple mesodermal genes. Furthermore, in animal explants, Sox17α prevents the induction of *Xbra* and *MyoD*, but not *Sox17β* or *Mixer*, in response to Xnr1. Therefore, VegT activation of *Sox17α* plays an important role in establishing a region of endoderm-specific gene expression in vegetal blastomeres. © 2001 Academic Press

Key Words: **endoderm; mesoderm; *Mixer*; *Nodal*; *Sox17*; *VegT*; *Xenopus*.**

INTRODUCTION

Segregation of the vertebrate embryo into the primary germ layers, endoderm, mesoderm, and neurectoderm, is the initial step in the generation of the diverse cell types of the adult organism. In *Xenopus*, formation of the germ layers is apparent prior to the gastrula stage with the complementary expression patterns of *Sox17α* in vegetal blastomeres, which form the future endoderm, and the adjacent and nonoverlapping expression of *Brachyury* (*Xbra*) in marginal blastomeres, which form the future mesoderm (Smith *et al.*, 1991; Hudson *et al.*, 1997). Despite the complementary expression patterns of these mesodermal and endodermal genes, a common set of regulatory factors controls the endogenous expression of both mesodermal and endodermal genes (reviewed in Yasuo and Lemaire, 2001). The mechanisms responsible for establishing a region of endodermal gene expression, spatially distinct from the mesoderm, have not been determined. Gaining an understanding of this process is important, for in addition to giving rise to the epithelial lining of the respiratory and digestive tracts, the prospective endoderm is a critical center for signaling and morphogenesis of the *Xenopus* embryo (Nieuwkoop, 1969, 1973; Winklbauer and Schurfeld, 1999).

At the onset of gastrulation, two related HMG-box transcription factors, Sox17α and Sox17β, are expressed throughout the vegetal region that forms the endoderm (Hudson *et al.*, 1997). In addition to their panendodermal expression, the *Sox17* genes are both necessary and sufficient for endodermal development (Hudson *et al.*, 1997). Several other transcription factors have been identified that regulate endoderm formation and are expressed with *Sox17*

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in the vegetal pole of the early gastrula. These factors include the homeobox genes *Mix.1*, *Mix.2*, *Mixer* (*Mxr*), *Mlk*/*Bix2*, and *Bix1*, *-3*, and *-4* (Rosa, 1989; Vize, 1996; Ecochard *et al.*, 1998; Henry and Melton, 1998; Tada *et al.*, 1998). However, unlike the exclusive expression of *Sox17* in the future endoderm, expression of *Mxr*, as well as the *Mix* and *Bix* genes, extends beyond the *Sox17* expression domain and overlaps with *Xbra* (Ecochard *et al.*, 1998; Lemaire *et al.*, 1998; Tada *et al.*, 1998). *Gata5*, another transcriptional regulator of endoderm, is expressed in a subset of the future endodermal cells at the early gastrula stage and is not expressed in supra-blastoporal cells (Weber *et al.*, 2000) that form the pharyngeal and head endoderm (Keller, 1991). Of these transcriptional regulators of early endodermal development, the *Sox17* genes are the only genes with panendodermal and no mesodermal expression at the early gastrula stage. Therefore, defining the mechanisms that generate restricted activation of *Sox17α* in vegetal blastomeres is essential for understanding the initiation of endodermal gene expression, as well as the spatial organization of the germ layers.

The *Sox17* genes and *Mxr* are expressed in explanted vegetal pole tissue soon after the midblastula transition and the *Sox17* genes, but not *Mxr*, are expressed in the isolated vegetal blastomeres of dissociated embryos (Hudson *et al.*, 1997; Clements *et al.*, 1999; Yasuo and Lemaire, 1999; Chang and Hemmati-Brivanlou, 2000). These observations indicate that vegetal determinants act at the midblastula transition to activate endodermal gene expression and that, in the case of *Sox17*, these determinants act cell autonomously. As a maternal mRNA localized to vegetal cells (Lustig *et al.*, 1996; Stennard *et al.*, 1996; Zhang and King, 1996; Horb and Thomsen, 1997), *VegT* is a potential regulator of *Mxr* and *Sox17* expression. *VegT* is necessary and sufficient for *Mxr* and *Sox17* expression in the gastrula (Casey *et al.*, 1999; Clements *et al.*, 1999; Yasuo and Lemaire, 1999; Chang and Hemmati-Brivanlou, 2000; Xanthos *et al.*, 2001) and *VegT* loss-of-function, via antisense oligonucleotide injection, results in embryos that do not form the endodermal germ layer (Zhang *et al.*, 1998; Xanthos *et al.*, 2001). Furthermore, *VegT* is required for the zygotic expression of several TGFβ ligands, including the *Nodal*-related genes (*Xnr1*, *-2*, *-4*, *-5*, *-6*) and *Derriere*, which have been implicated in mesodermal and endodermal development (Clements *et al.*, 1999; Kofron *et al.*, 1999; Yasuo and Lemaire, 1999; Chang and Hemmati-Brivanlou, 2000).

TGFβ signals are critical for the establishment and patterning of embryonic endoderm. A truncated Activin type II receptor that inhibits signaling by several TGFβ ligands (Hemmati-Brivanlou and Melton, 1992) blocks the endogenous expression of endodermal markers, demonstrating the requirement for TGFβ signaling in endodermal development (Gamer and Wright, 1995; Henry *et al.*, 1996; Yasuo and Lemaire, 1999; Chang and Hemmati-Brivanlou, 2000; Weber *et al.*, 2000). Genetic analyses in the mouse and zebrafish demonstrate an essential role for *Nodal*-related genes in endodermal development. Mice mutant for *Nodal* and zebrafish mutant for both *squint* and *cyclops*, two *Nodal*-related genes, lack all endodermal derivatives (Conlon *et al.*, 1994; Feldman *et al.*, 1998; Rebagliati *et al.*, 1998; Sampath *et al.*, 1998). In *Xenopus*, expression of a mutated Xnr2 ligand, predicted to specifically inhibit the activity of endogenous Nodal proteins, reduces the endogenous expression of early endodermal genes (Osada and Wright, 1999). These studies in *Xenopus*, mouse, and zebrafish support a central role for Nodal signaling in establishing embryonic endoderm (reviewed in Schier and Shen, 2000). Six *Nodal*-related genes (*Xnr1–6*) have been isolated from *Xenopus*, and all but *Xnr3* are expressed in vegetal cells of the blastula (Jones *et al.*, 1995; Smith *et al.*, 1995; Joseph and Melton, 1997; Agius *et al.*, 2000; Takahashi *et al.*, 2000) and have the ability to induce endodermal gene expression (Clements *et al.*, 1999; Osada and Wright, 1999; Yasuo and Lemaire, 1999; Takahashi *et al.*, 2000).

In current models of *Xenopus* endoderm formation, it is proposed that VegT initiates endodermal development by activating the vegetal expression of endoderm-specific transcription factors together with *Nodal*-related genes, and once expressed these genes further regulate endodermal gene expression (Clements *et al.*, 1999; Kofron *et al.*, 1999; Yasuo and Lemaire, 1999; Chang and Hemmati-Brivanlou, 2000). Nodal-related signals activate zygotic expression of *VegT* (Lustig *et al.*, 1996; Stennard *et al.*, 1996, 1999; Horb and Thomsen, 1997), as well as maintain their own expression (Jones *et al.*, 1995; Osada and Wright, 1999; Agius *et al.*, 2000; Takahashi *et al.*, 2000). The reciprocal regulatory interactions of *VegT* and *Nodal*-related genes represent a positive feedback loop for the initiation and maintenance of endodermal gene expression in vegetal cells. The interaction of these genes raises questions about their roles in activating individual endodermal genes, and whether direct or indirect mechanisms are involved. Furthermore, in addition to their role in endoderm formation, *VegT* and *Nodal*-related genes are necessary and sufficient for mesoderm formation (Zhang *et al.*, 1998; Kofron *et al.*, 1999; Piccolo *et al.*, 1999; Agius *et al.*, 2000). How *VegT* and *Nodal*-related genes regulate the development of both mesoderm and endoderm, lineages that are functionally and spatially distinct, is an important question that remains to be answered.

Here, we show that the onset of *Sox17α* expression at the midblastula transition is activated directly by VegT and subsequently maintained by Nodal signals. Using specific inhibitiors of VegT and Nodal, we demonstrate a requirement for both activities for *Sox17α* expression at the gastrula stage, but only VegT is required for the initiation of *Sox17α* expression at the midblastula transition. Given that *Nodal*-related genes can induce both endoderm and mesoderm, we propose that VegT activation of *Sox17α* expression at the midblastula transition may prevent the vegetal induction of mesodermal genes by Nodal signals. Misexpression of Sox17α in the marginal zone inhibits endogenous mesodermal gene expression, demonstrating that Sox17α is incompatible with mesodermal gene expression.

In addition, Sox17α inhibits the induction of *Xbra* and *MyoD*, but not *Sox17β* or *Mxr*, by Xnr1. These experiments indicate that Sox17α can bias the response to Nodal signals toward an endodermal response. Therefore, the results suggest that VegT activation of *Sox17α* in vegetal cells defines the vegetal endodermal domain by preventing the activation of mesodermal genes in response to Nodal signals.

MATERIALS AND METHODS

Embryos and Microinjection

Embryos were collected, fertilized, injected, and cultured as previously described (Yao and Kessler, 1999), and embryonic stage was determined according to Nieuwkoop and Faber (1967). Explants were prepared using a Gastromaster microsurgery instrument (Xenotek Engineering). Capped, *in vitro* transcribed RNA for microinjection was synthesized using the Message Machine kit (Ambion) programmed with linearized DNA template, and 10 nl of RNA solution was injected. Sox17α was obtained by PCR amplification of the complete coding region with Vent polymerase and subcloning into pCS2+ (Rupp *et al.*, 1994). A VegT construct lacking the 3′-UTR (pCS2-VegTΔUTR) was constructed by PCR amplification of the VegT coding region from pCS2-Brat (Horb and Thomsen, 1997) and subcloning into pCS2+. The Eng-VegT construct used in this study is identical to pCS2-Brat-En^R (Horb and Thomsen, 1997). Templates for *in vitro* transcription were pCS2-VegTΔUTR (this study), pCS2-Xnr1 (Sampath *et al.*, 1997), pCS2-Cer-S (Piccolo *et al.*, 1999), pCS2-Eng-VegT/pCS2-Brat-En^R (Horb and Thomsen, 1997), and pCS2-Sox17α (this study). For cycloheximide experiments, explants were cultured in 0.5× MMR supplemented with 5 μg/ml cycloheximide (Sigma). For lineage labeling experiments, Oregon Green-dextran (10,000 MW; Molecular Probes) was combined with *in vitro* transcribed RNA at a final concentration of 2.5 mg/ml for injection.

Reverse Transcription–Polymerase Chain Reaction

Total RNA was isolated from explants and embryos using the RNAqueous kit (Ambion), and cDNA synthesis and PCR were performed as described (Wilson and Melton, 1994). Radiolabeled PCR products were resolved on 5% native polyacrylamide gels. PCR primers and cycle numbers were as described for EF1α and Xbra (Wilson and Melton, 1994), ODC (Agius *et al.*, 2000), and MyoD (Rupp and Weintraub, 1991). PCR primers and cycle numbers for Mxr, Sox17α, and Sox17β were: Mxr, upstream 5′-CACCAGCCCAGCACTTAACC-3′, downstream 5′-CAATGTCACATCAACTGAAG-3′, 25 cycles; Sox17α, upstream 5′-CAGGTGAAGAGGATGAAGAG-3′, downstream 5′-GCTGGAGATGTGAAGAACAC-3′, 22–25 cycles; Sox17β, upstream 5′ CAGGTGAAGAGGATGAAGAG-3′, downstream 5′-CATTGAGTTGTGGCCCTCAA-3′, 25 cycles.

In Situ Hybridization

Whole-mount *in situ* hybridization was performed as described (Sive *et al.*, 2000). Hybridized probe was detected using alkaline phosphatase conjugated to anti-digoxigenin Fab fragments (Boehringer Mannheim) and BMpurple (Boehringer Mannheim) as substrate for color development. Section *in situ* hybridization was performed essentially as described (Lemaire and Gurdon, 1994) with the following modifications. Hybridization was performed at 56°C with 1 μg/ml probe. Subsequent washes were performed at room temperature and RNase treatment was omitted. Antisense probes were synthesized from linearized plasmid DNA using the Megascript kit (Ambion) supplemented with 2 mM digoxigenin-11-UTP (Boehringer Mannheim). Sox17α and Mxr templates were obtained by RT-PCR amplification of gastrula mRNA and subcloning of complete coding regions into pT7blue (Novagen). Other *in situ* probes were synthesized from linearized pGEM-Xbra (Wilson and Melton, 1994), pCS2-Cer (Bouwmeester *et al.*, 1996), pBS-Dlx3 (Feledy *et al.*, 1999), pBS-Endodermin (Sasai *et al.*, 1996), pGEM-Gsc (Cho *et al.*, 1991), pGEM-Mix.1 (Rosa, 1989), pBS-MyoD (Rupp *et al.*, 1994), pBS-Opl (Kuo *et al.*, 1998), and pGEM-Xwnt8 (Sokol *et al.*, 1991).

RESULTS

Endodermal Gene Expression in the Gastrula Is Dependent on VegT and Nodal Function

VegT loss-of-function has been accomplished with an antisense oligonucleotide that depletes maternal VegT mRNA (Zhang *et al.*, 1998) or with an Engrailed repressor-VegT fusion protein (Eng-VegT) that represses target genes normally activated by VegT (Horb and Thomsen, 1997). Although both approaches resulted in a similar failure to form mesoderm or axial structures, the reported effects of antisense depletion and Eng-VegT on the expression of the endodermal genes *Mxr* and *Sox17α* are contradictory. Although Eng-VegT was reported to inhibit the expression of several endodermal genes in a dose-dependent manner, *Mxr* expression was not inhibited and the inhibition of *Sox17α* was incomplete (Chang and Hemmati-Brivanlou, 2000), contrasting with the near complete loss of *Mxr* and *Sox17α* expression in embryos depleted of *VegT* mRNA (Xanthos *et al.*, 2001). It may be that the RT-PCR assay used in the Eng-VegT studies detected residual levels of *Sox17α* and *Mxr* expression, thus underestimating the effects of Eng-VegT on endoderm formation.

To clarify the requirement for VegT function in early endodermal gene expression, *Mxr* and *Sox17α* expression was analyzed by *in situ* hybridization of Eng-VegT-injected embryos. Eng-VegT mRNA was injected into the vegetal pole of each blastomere at the four-cell stage. Embryos were harvested at the gastrula stage and the expression of *Mxr*, *Sox17α*, and *Xbra* was analyzed in histological sections by *in situ* hybridization. As previously shown (Horb and Thomsen, 1997), injection of Eng-VegT completely inhibited the expression of *Xbra* (Figs. 1G and 1H). In addition, the expression of both *Sox17α* (Figs. 1A and 1B) and *Mxr* (Figs. 1D and 1E) was nearly eliminated by Eng-VegT, indicating that VegT function is required for the expression of *Mxr* and *Sox17α* throughout the prospective endoderm. The presence of scattered *Mxr*- and *Sox17α*-expressing vegetal cells may be due to mosaic distribution of Eng-VegT which, as a transcriptional regulator, is likely to act in a

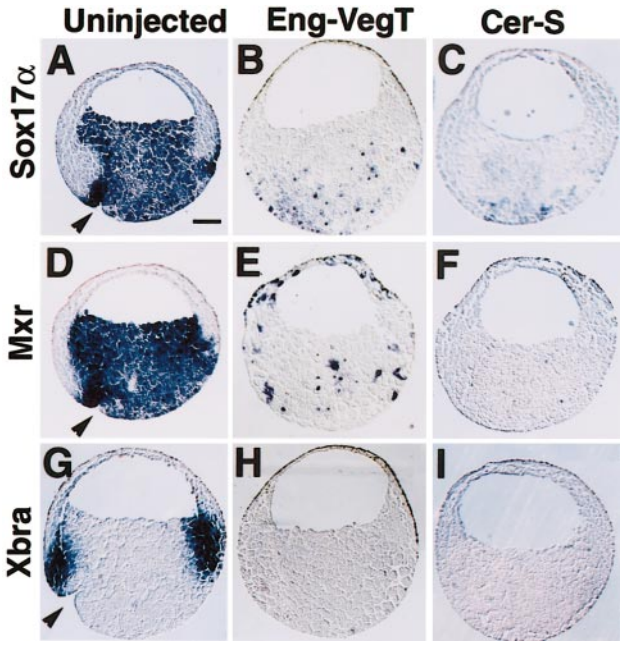


FIG. 1. VegT and Nodal function are required for endodermal gene expression. At the four-cell stage, each blastomere was injected vegetally with 300 pg of Eng-VegT mRNA or 100 pg of Cer-S mRNA. Uninjected (A, D, G), Eng-VegT-injected (B, E, H), and Cer-S-injected (C, F, I) embryos were fixed at the early gastrula stage (stage 10.25) and sagittal sections were analyzed by *in situ* hybridization for the expression of *Sox17α* (A–C), *Mxr* (D–F), and *Xbra* (G–I). Arrowheads indicate the dorsal blastopore lip. Scale bar, 0.25 mm.

cell-autonomous manner. We also observed a few *Mxr*-positive animal pole cells in Eng-VegT-injected embryos (Fig. 1E), suggesting that Eng-VegT may repress targets that negatively regulate *Mxr* expression in the animal pole. The expression of *Mix.1* (Rosa, 1989) and *Cerberus* (Bouwmeester *et al.*, 1996), additional genes expressed in the prospective endoderm, was also inhibited by Eng-VegT (data not shown). The dramatic reduction in *Mxr* and *Sox17α* expression resulting from Eng-VegT injection demonstrates that VegT function is essential for endodermal gene expression in the gastrula, consistent with the results of antisense depletion of *VegT* (Xanthos *et al.*, 2001).

Eng-VegT inhibited endodermal gene expression in vegetal blastomeres and did not induce ectopic expression of mesodermal genes in these same cells, suggesting that the Eng-VegT-injected cells are neither endodermal nor mesodermal. To determine whether these cells adopt epidermal or neural fates at the gastrula stage, the expression of *Dlx3* (non-neural ectoderm; Feledy *et al.*, 1999) and *Opl* (neural plate; Kuo *et al.*, 1998) was examined. Vegetal expression of *Dlx3* or *Opl* was not observed in Eng-VegT-injected embryos (data not shown). The absence of ectopic ectodermal, neural, or mesodermal gene expression in Eng-VegT-injected cells suggests that these cells have not adopted an alternative fate at the gastrula stage. However, *VegT* loss-of-function by antisense depletion has been shown to result in a conversion of vegetal cells into ectoderm at the tailbud stage (Zhang *et al.*, 1998). The absence of ectodermal gene expression with Eng-VegT injection may indicate that vegetal cells lacking VegT function do not adopt ectodermal fate until after the gastrula stage. Alternatively, Eng-VegT may be incompatible with the expression of ectodermal genes.

Given that VegT regulates the expression of *Nodal*-related genes and that Nodal function is required for formation of the endodermal lineage, the dependence of *Mxr* and *Sox17α* expression on Nodal function was examined. To inhibit endogenous Nodal-related proteins a truncated form Cerberus, a secreted antagonist of Nodal function (Bouw-

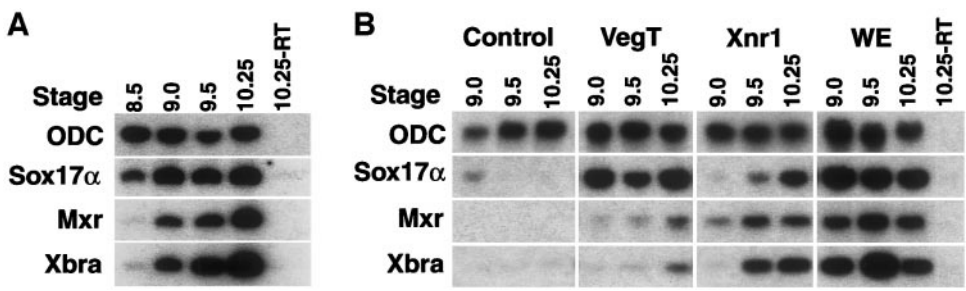


FIG. 2. *Sox17α* and *Mxr* differ in the onset of expression. (A) *Sox17α* is expressed at the midblastula transition prior to the expression of *Mxr* and *Xbra*. Intact embryos were harvested at stages 8.5, 9.0, 9.5, and 10.25 for RT-PCR analysis of *Sox17α*, *Mxr*, and *Xbra* expression. (B) *Sox17α* and *Mxr* differ in the onset of expression in response to VegT and Xnr1. At the two-cell stage, the animal pole was injected with 300 pg of VegT or 500 pg of Xnr1 mRNA. Animal explants were prepared at stage 8 from uninjected (Control) or injected embryos and both explants and whole embryos (WE) were harvested at stages 9.0, 9.5, and 10.25 for RT-PCR analysis of *Sox17α*, *Mxr*, and *Xbra* expression. *Ornithine decarboxylase* (*ODC*) served as a control for RNA recovery and loading. Stage 10.25 whole embryo mRNA was used in a cDNA synthesis reaction without the addition of reverse transcriptase to control for PCR contamination (10.25-RT).

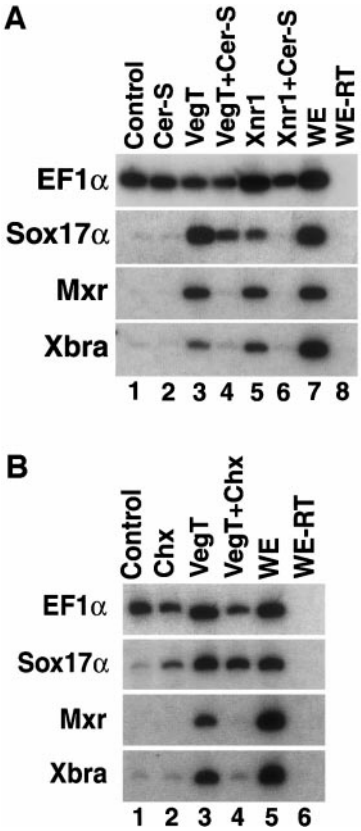


FIG. 3. VegT activation of *Sox17α* is not dependent on Nodal signals or protein synthesis. (A) At the one-cell stage, the animal pole was injected with 1 ng of Cer-S mRNA and, at the two-cell stage, 300 pg of VegT or 500 pg of Xnr1 mRNA was injected. Animal explants prepared at the blastula stage were harvested at the gastrula stage for RT-PCR analysis of *Sox17α*, *Mxr*, and *Xbra* expression. (B) At the two-cell stage, the animal pole was injected with 300 pg of VegT mRNA. Animal explants prepared at stage 7 were cultured with or without cycloheximide (Chx, 5 μg/ml) and were harvested at stage 10.25 for RT-PCR analysis. *EF1α* served as a control for RNA recovery and loading. Whole embryos served as positive control (WE) and an identical reaction without reverse transcriptase controlled for PCR contamination (WE-RT).

meester *et al.*, 1996), was misexpressed in the embryo. This truncated form of Cerberus (Cerberus-Short, Cer-S) specifically binds to extracellular Nodal proteins and inhibits signaling by Xnr1, Xnr2, Xnr4, Xnr5, and Xnr6, but does not inhibit other TGFβ-related proteins, including Activin, BMP4, Derriere, or Vg1 (Piccolo *et al.*, 1999; Agius *et al.*, 2000; Takahashi *et al.*, 2000). Cer-S mRNA was injected into the vegetal pole of each blastomere at the four-cell stage and embryos were harvested at the gastrula stage for analysis of *Mxr*, *Sox17α*, and *Xbra* expression by section *in situ* hybridization. Cer-S nearly eliminated *Sox17α* expression (Fig. 1C) and completely inhibited the expression of *Mxr* (Fig. 1F). Therefore, Nodal function is required for the expression of *Mxr* and *Sox17α* in the prospective endoderm at the gastrula stage. In addition, *Xbra* expression was completely inhibited by Cer-S (Fig. 1I), consistent with previous studies (Piccolo *et al.*, 1999; Agius *et al.*, 2000). The ability of Cer-S to inhibit *Sox17α* and *Mxr* expression is consistent with the ability of a dominant-negative cleavage mutant of Xnr2 to reduce the expression of endodermal markers, including *Mxr* and *Sox17α*, at the gastrula stage (Osada and Wright, 1999). Using a RT-PCR assay, Piccolo *et al.* (1999) reported that *Sox17* expression was unaffected by Cer-S injection and this contrasts with the near complete elimination of *Sox17α* expression in our *in situ* hybridization analysis. As suggested above, the RT-PCR assay may have detected residual levels of *Sox17α* expression, thus underestimating the effects of Cer-S on endoderm formation. Cer-S-injected embryos were also examined for the expression of *Dlx3* and *Opl* and no ectopic expression was observed in vegetal cells (data not shown), suggesting that these cells do not adopt ectodermal or neural fates at the gastrula stage.

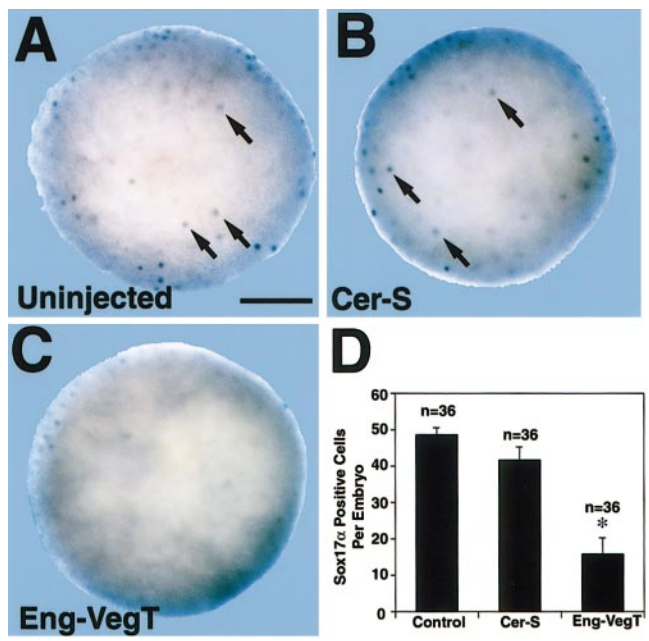


FIG. 4. Initiation of *Sox17α* expression at the midblastula transition requires VegT function, but not Nodal. At the four-cell stage, each blastomere was injected vegetally with 300 pg of Eng-VegT mRNA or 100 pg of Cer-S mRNA. Uninjected (A), Cer-S-injected (B), and Eng-VegT-injected (C) embryos were harvested at stage 8.5 and analyzed for *Sox17α* expression by *in situ* hybridization. *Sox17α* expression was unaffected by Cer-S (B), but greatly reduced by Eng-VegT (C). Vegetal views with arrows indicating the perinuclear staining of *Sox17α*-positive cells are shown. Scale bar, 0.25 mm. (D) Quantitation of *Sox17α*-positive cells. The mean and standard error of the number of *Sox17α*-positive cells per embryo ($n = 36$) are shown. Statistical significance was assessed using the Student's *t* test (*, $P < 0.001$).

In addition to changes in endodermal gene expression, embryos injected with Cer-S or Eng-VegT exhibited similar changes in morphology, as compared to uninjected controls. In both cases, injected embryos failed to gastrulate and did not form a blastopore lip (Fig. 1), consistent with a failure to form embryonic endoderm and mesoderm. Furthermore, Cer-S- and Eng-VegT-injected embryos had a rounded blastocoel floor at the gastrula stage (Figs. 1B, 1C, 1E, 1F, 1H, and 1I), similar to the blastocoel floor of the early blastula and unlike the flattened blastocoel floor of the gastrula (Figs. 1A, 1D, and 1G). The abnormal blastocoel morphology of the injected embryos may result from a failure to undergo vegetal rotation, a morphogenetic movement of the vegetal pole that occurs in the early gastrula (Winklbauer and Schurfeld, 1999). Therefore, inhibition of VegT or Nodal function may disrupt the morphogenetic behavior of the prospective endoderm, in addition to blocking activation of endodermal gene expression.

Initiation of Mxr and Sox17α Expression in Response to VegT and Xnr1

The results discussed above demonstrate an essential role for *VegT* and *Nodal*-related genes in the expression of endodermal genes at the gastrula stage. To further define the role of *VegT* and *Nodal*-related genes in the activation of endodermal gene expression, the onset of *Mxr* and *Sox17α* expression was examined in intact embryos and in animal explants injected with VegT or Xnr1 mRNA. Gene expression was analyzed by RT-PCR in intact embryos collected at the midblastula transition and at 45-min intervals thereafter (Fig. 2A). *Sox17α* expression was detected immediately after the midblastula transition (stage 8.5) and strong expression was observed through the early gastrula stage (stage 10.25). In contrast, *Mxr* expression was not detected until the late blastula stage (stage 9.0), 45 min later than the onset of *Sox17α* expression. *Mxr* expression increased gradually, not reaching maximal levels until the beginning of gastrulation. The delayed onset and gradual increase of *Mxr* expression was strikingly similar to the expression profile of *Xbra*. So while *Mxr* and *Sox17α* have similar spatial expression patterns, the delay in the onset of *Mxr* expression relative to *Sox17α* suggests a difference in the mechanisms regulating these genes.

The onset of *Mxr* and *Sox17α* expression was also examined in animal explants injected with VegT or Xnr1. At the two-cell stage, the animal pole was injected with VegT or Xnr1 mRNA and animal explants, prepared at the early blastula stage, were harvested for RT-PCR analysis at 45-min intervals beginning at stage 9.0 (Fig. 2B). Similar to the difference in the onset of endogenous expression, the onset of *Mxr* and *Sox17α* expression differs significantly in response to VegT and Xnr1 in explants. VegT induced strong expression of *Sox17α* at the earliest point examined (stage 9.0) and this level was maintained through the early gastrula stage. Xnr1 induced little or no *Sox17α* expression at early points and strong expression was not detected until the early gastrula stage. In contrast to the response of *Sox17α*, *Mxr* induction by VegT was not apparent until the early gastrula stage, while Xnr1 induced *Mxr* expression at blastula stages. The response of *Xbra* was similar to that observed for *Mxr*, with activation by VegT at the early gastrula stage and activation by Xnr1 at blastula stages. The results suggest that initiation of *Sox17α* expression at the midblastula transition is regulated by maternal VegT, but not by Nodal-related factors. In addition, the initiation of *Mxr* expression may be more dependent on Nodal-related factors than VegT.

VegT Activation of Sox17α Is Direct and Independent of Nodal Signaling

The expression of *Sox17α* in the prospective endoderm is dependent on both VegT and Nodal function, but the onset of *Sox17α* expression in response to these factors differs. To examine the interaction of VegT and Nodal signals in the regulation of endodermal gene expression, the dependence of VegT on Nodal signaling in the activation of *Mxr* and *Sox17α* expression was determined. At the two-cell stage, VegT, Cer-S, or a combination of both mRNAs was injected into the animal pole and the expression of *Mxr*, *Sox17α*, and *Xbra* was assessed by RT-PCR at the gastrula stage. As expected, VegT induced *Sox17α*, *Mxr*, and *Xbra* expression (Fig. 3A, lane 3). Coexpression of Cer-S and VegT completely blocked activation of *Mxr* and *Xbra*, while *Sox17α* induction was only slightly reduced (Fig. 3A, lane 4). As a positive control, Cer-S inhibited the induction of all three genes in response to Xnr1 overexpression (Fig. 3A, lanes 5, 6). The ability of VegT to activate *Sox17α* expression in the absence of Nodal signaling suggests that VegT may be a direct activator of *Sox17α* expression. In contrast, Nodal function is required for the induction of *Mxr* by VegT, suggesting that activation of *Mxr* expression by VegT occurs indirectly, via Nodal signals.

A direct target, or immediate-early response gene, is defined as a transcriptional target that can be activated or repressed by a given regulatory factor without a requirement for *de novo* protein synthesis. Several observations suggest that VegT is a direct activator of *Sox17α*. *Sox17α* is expressed in embryos treated with cycloheximide, a translation inhibitor, suggesting that *Sox17α* is a direct target of maternal determinants (Yasuo and Lemaire, 1999). *Sox17α* is expressed immediately following the midblastula transition in the vegetal pole of intact embryos and in VegT-injected animal explants (Figs. 2A and 2B; Clements *et al.*, 1999; Yasuo and Lemaire, 1999). In addition, VegT can activate *Sox17α* in dissociated explants where cell signaling is disrupted (Clements *et al.*, 1999; Yasuo and Lemaire, 1999; and data not shown). Finally, as shown above, VegT can activate *Sox17α* expression independent of Nodal signaling. To determine whether *Mxr* and *Sox17α* are direct targets of VegT, the ability of VegT to activate *Mxr*, *Sox17α*, and *Xbra* in the presence of cycloheximide was examined (Fig. 3B). Embryos were injected at the two-cell stage with

VegT mRNA. Animal explants, prepared before the midblastula transition (stage 7), were cultured in the presence of cycloheximide, and were analyzed by RT-PCR at the gastrula stage for the expression of *Mxr*, *Sox17α*, and *Xbra*. VegT induced strong expression of *Mxr*, *Sox17α*, and *Xbra* (Fig. 3B, lane 3) and the addition of cycloheximide resulted in a complete block of *Mxr* and *Xbra* expression, but did not block induction of *Sox17α* (Fig. 3B, lane 4). In this experiment, treatment with cycloheximide alone resulted in a low level of *Sox17α* expression (Fig. 3B, lane 2) and quantitation confirmed that *Sox17α* levels were significantly higher in response to VegT plus cycloheximide (data not shown), indicating that VegT activates *Sox17α* without ongoing protein synthesis. The insensitivity of *Sox17α* induction to cycloheximide suggests that activation of *Sox17α* transcription in response to VegT is mediated by proteins present at the midblastula stage and does not require synthesis of intervening regulators. The results indicate that *Sox17α* is an immediate-early target of VegT, suggesting that VegT may be responsible for the direct transcriptional activation of *Sox17α* at the midblastula transition. In contrast, activation of *Mxr* and *Xbra* expression by VegT requires the translation of additional proteins, likely to include Nodal-related proteins, that act with or downstream of VegT to mediate induction.

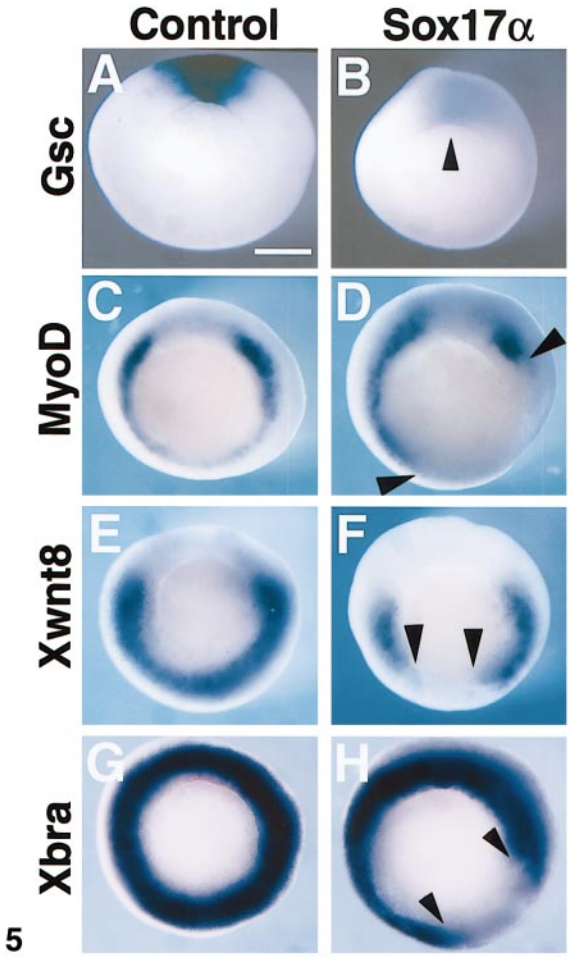
VegT Is Required for Initiation of Sox17α Expression at the Midblastula Transition

The ability of VegT to induce *Sox17α* expression during the blastula stages as an immediate-early target gene suggests that VegT directly activates *Sox17α* expression at the midblastula transition. Consistent with direct activation, VegT can induce *Sox17α* in the presence of Cer-S, suggesting that VegT functions independent of Nodal signaling to activate *Sox17α*. To determine whether the initiation of endogenous *Sox17α* expression is dependent on VegT, but not Nodal, the requirement for VegT and Nodal function in the onset of *Sox17α* expression at the midblastula transition was examined. Eng-VegT or Cer-S mRNA was injected into the vegetal pole of each blastomere at the four-cell stage. Embryos were harvested at stage 8.5 and the expression of *Sox17α* was analyzed by whole-mount *in situ* hybridization (Fig. 4). In contrast to the strong, uniform expression throughout the vegetal pole of the gastrula (see Fig. 1), *Sox17α* expression at the midblastula stage is detected as a perinuclear staining of the vegetal cells (Fig. 4A). This perinuclear localization of transcripts has been observed for other genes as transcription is initiated and may reflect an intermediate step in mRNA processing. *Sox17α* expression was unaffected by Cer-S injection (Fig. 4B), with the number of *Sox17α*-positive cells and the staining intensity indistinguishable from uninjected controls (Fig. 4D). In siblings analyzed at the gastrula stage, *Sox17α* expression was nearly eliminated, confirming that the Cer-S injection was effective (data not shown). In contrast, *Sox17α* expression was greatly reduced in Eng-VegT-injected embryos at the midblastula transition (Fig. 4C). Five-fold fewer *Sox17α*-positive cells were observed with Eng-VegT injection as compared to uninjected controls (Fig. 4D). Following the onset of expression at the midblastula transition, *Sox17α* expression rapidly becomes dependent on Nodal signaling and at stage 9, 45 min after the midblastula transition, a reduction in *Sox17α* expression is observed in response to Cer-S injection (data not shown). The result suggests that the initiation of *Sox17α* expression at the midblastula transition is dependent on VegT function, but not Nodal signaling. Given the dependence on Nodal signaling at later stages, the data suggest that the onset of *Sox17α* expression at the midblastula transition is regulated by VegT, and soon afterwards the expression of *Sox17α* is maintained by Nodal signals.

Sox17α Inhibits Mesodermal Gene Expression

Endodermal and mesodermal genes are expressed in adjacent domains in the gastrula, yet the expression of both classes of genes is dependent on Nodal signals. Although Nodal signals are active in vegetal cells (Jones *et al.*, 1995; Agius *et al.*, 2000; Faure *et al.*, 2000) and endodermal genes respond to these signals, mesodermal genes do not. The mechanisms that prevent mesodermal gene expression in vegetal cells in response to Nodal are not understood. One possibility is that direct activation of *Sox17α* by VegT at the midblastula transition establishes the vegetal endodermal domain by preventing the induction of mesodermal genes. The validity of this idea was tested by examining mesodermal gene expression following misexpression of Sox17α in the marginal zone. At the four-cell stage, a single blastomere was injected in the marginal zone with Sox17α mRNA and embryos were harvested at the gastrula stage for *in situ* hybridization (Fig. 5). Sox17α misexpression in the marginal zone resulted in a significant reduction of *Goosecoid* expression (Figs. 5A and 5B) and a gap in the expression domains of *MyoD* (Figs. 5C and 5D), *Xwnt8* (Figs. 5E and 5F), and *Xbra* (Figs. 5G and 5H), indicating that Sox17α misexpression inhibits the expression of these mesodermal genes. Sox17α misexpression caused a gap in *Xbra* expression in most of the embryos analyzed (79%, $n = 53$), while *Xbra* expression was normal in nearly all control embryos.

To determine the cell-autonomy of Sox17α inhibition of mesodermal genes, the spatial relation of the Sox17α-injected cells with the gap in *Xbra* expression was determined. At the four-cell stage, a single blastomere was injected in the marginal zone with the fluorescent lineage tracer Oregon Green-Dextran (OGD) alone, or in combination with Sox17α mRNA. Embryos with marginal zone fluorescence were harvested at the gastrula stage for *in situ* hybridization. As above, Sox17α inhibited *Xbra* expression in the marginal zone (Figs. 6A–6D). The spatial relation of the Sox17α-injected cells with the gap in *Xbra* expression was determined by visualizing the OGD-positive cells following *in situ* hybridization. In every case, the position of the Sox17α-expressing, OGD-positive cells corresponded



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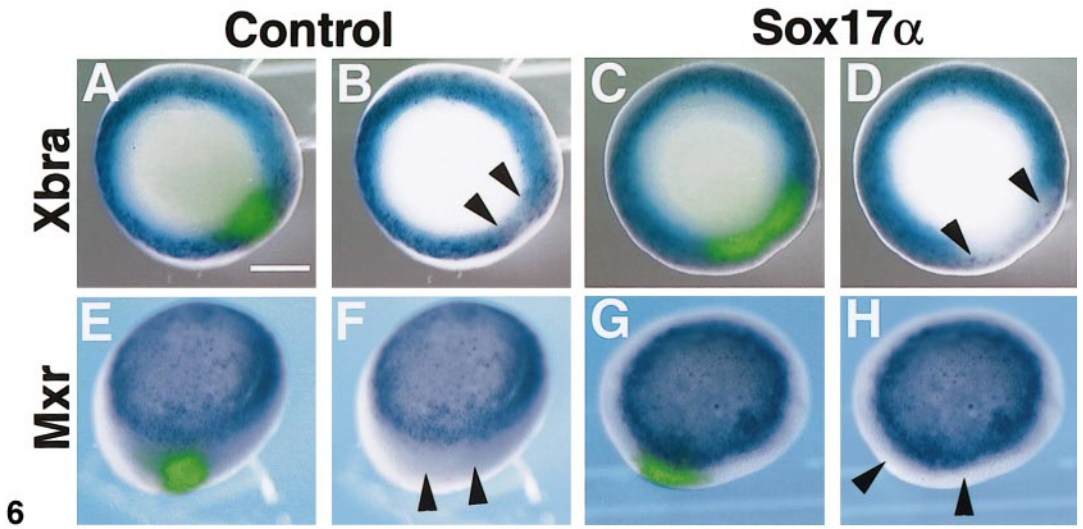

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precisely to the gap in *Xbra* expression (Figs. 6C and 6D), consistent with a cell-autonomous inhibition of *Xbra* expression by Sox17α.

The results suggest that *Sox17α* expression is incompatible with mesodermal gene expression. One potential mechanism for this activity of Sox17α is the conversion of marginal zone cells into endoderm. To assess this possibility, Sox17α-injected embryos were analyzed for ectopic expression of the endodermal genes *Mxr* and *Endodermin* (*Edd*; Sasai *et al.*, 1996). Sox17α misexpression in the marginal zone did not result in ectopic activation of *Mxr* (Figs. 6E–6H) or *Edd* (data not shown). Although a high percentage of Sox17α-injected embryos had a gap in the *Xbra* expression domain, none of the Sox17α-injected embryos displayed ectopic expression of *Mxr* (n = 37) or *Edd* (n = 25). The absence of endodermal gene expression in Sox17α-expressing marginal zone cells suggests that loss of mesodermal gene expression is not due to a conversion from mesodermal to endodermal fate. To further assess the fate of Sox17α-expressing marginal cells, the expression of *Opl* (neural plate) and *Dlx3* (non-neural ectoderm) was examined. Ectopic expression of neither marker was detected in the marginal zone of Sox17α-injected embryos (data not shown), indicating that these cells do not adopt neural or ectodermal fates at the gastrula stage. Therefore, the inhibition of mesodermal genes by Sox17α is not accompanied by an upregulation of endodermal genes, suggesting that one aspect of Sox17α function in establishing the endodermal fate of vegetal cells is the inhibition of mesodermal gene expression.

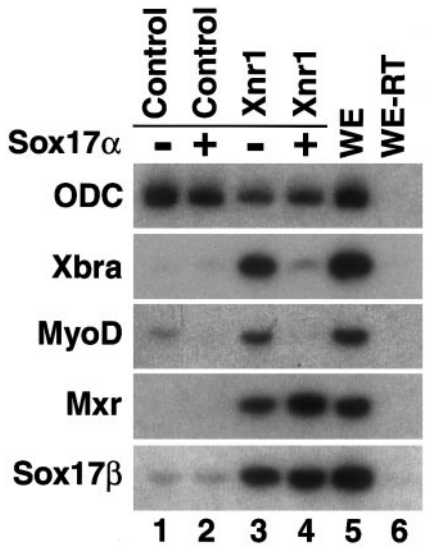

FIG. 7. Sox17α alters the response to Xnr1. At the one-cell stage, the animal pole was injected with 250 pg of Sox17α mRNA and at the two-cell stage, 30 pg of Xnr1 mRNA was injected. Animal explants were prepared at the blastula stage and harvested at the gastrula stage for RT-PCR analysis of *Xbra*, *MyoD*, *Mxr*, and *Sox17β* expression. While Xnr1 induced both mesodermal and endodermal genes, Sox17α coexpression prevented the induction of the mesodermal genes without affecting the response of the endodermal genes. *ODC* served as a control for RNA recovery and loading. Whole embryos served as a positive control (WE) and an identical reaction without reverse transcriptase controlled for PCR contamination (WE-RT).

Sox17α Alters the Response to Nodal Signaling

The inhibition of endogenous mesodermal gene expression by Sox17α prompted an examination of the ability of Sox17α to interfere with the mesodermal response to Nodal signals. Xnr1 mRNA was injected alone, or in combination with Sox17α mRNA, into the animal pole, and explants were analyzed by RT-PCR for endodermal and mesodermal gene expression at the gastrula stage (Fig. 7). While Xnr1 alone induced the expression of *Xbra*, *MyoD*, *Mxr*, and *Sox17β* (Fig. 7, lane 3), coexpression of Sox17α with Xnr1 inhibited the activation of the mesodermal genes *Xbra* and *MyoD* without affecting the activation of the endodermal genes (Fig. 7, lane 4). Sox17α alone did not induce expression of the endodermal or mesodermal genes (Fig. 7, lane 2), consistent with the inability of Sox17α to induce early endodermal genes (Henry and Melton, 1998). Therefore, Sox17α can alter the transcriptional response to Nodal signals, such that endodermal, but not mesodermal genes, are induced. Furthermore, the results suggest that the direct

FIG. 5. Sox17α inhibits mesodermal gene expression in the marginal zone. At the four-cell stage, a single blastomere was injected in the marginal zone with 500 pg of Sox17α mRNA (B, D, F, H). Uninjected (Control) and injected embryos were collected at stage 10.25 for *in situ* hybridization analysis of *Goosecoid* (*Gsc*; A, B), *MyoD* (C, D), *Xwnt8* (E, F), and *Xbra* (G, H) expression (vegetal views, dorsal up). Sox17α misexpression resulted in reduction of *Goosecoid* (B) expression and a gap in the expression domains of *MyoD* (D), *Xwnt8* (F), and *Xbra* (H). Arrowheads indicate the regions of reduced gene expression. Scale bar, 0.25 mm.

FIG. 6. Cell autonomous inhibition of mesodermal gene expression by Sox17α without ectopic endodermal gene expression. At the four-cell stage, a single blastomere was injected in the marginal zone with the fluorescent lineage marker, Oregon Green-dextran (OGD), alone (A, B, E, F), or together with 500 pg of Sox17α mRNA (C, D, G, H). Injected embryos were collected at stage 10.25 for *in situ* hybridization analysis of *Xbra* (A–D) and *Mxr* (E–H) expression. Vegetal views (dorsal up) of the *in situ* staining pattern (B, D, F, H) or merged images of OGD-positive cells and the *in situ* stain (A, C, E, G) are shown. The position of OGD-positive, Sox17α-expressing cells corresponds precisely to the gap in *Xbra* expression, but no ectopic *Mxr* expression was observed. Arrowheads indicate the position of OGD-positive cells. Scale bar, 0.25 mm.

activation of *Sox17α* by VegT in vegetal cells, prior to the expression of other endodermal or mesodermal genes, plays an important role in establishing the vegetal endodermal domain by preventing the expression of mesodermal genes in response to Nodal signals.

DISCUSSION

In recent years, much effort has been directed at defining the molecular events that regulate formation of the germ layers. Dominant-negative and loss-of-function approaches have implicated *Nodal*-related genes and *VegT* as critical regulators of endodermal development. In addition, functional and expression screens have identified genes expressed in the prospective endoderm of the early gastrula, and these are likely to be important regulatory targets of the vegetal determinants that establish the endodermal germ layer. One challenge in defining the mechanisms of endodermal specification is to determine the specific roles of VegT and Nodal signals in the regulation of individual endodermal target genes. In this work, we demonstrate that VegT initiates *Sox17α* expression as an immediate-early target at the midblastula transition and that *Sox17α* expression is subsequently maintained by Nodal signals. In contrast, the regulation of *Mxr* differs, with VegT acting indirectly in a Nodal-dependent manner to activate *Mxr* expression. Furthermore, our studies address an additional important question. How do Nodal-related factors induce the development of both the mesodermal and endodermal germ layers, tissues that are functionally and spatially distinct? Here, we propose that VegT activation of *Sox17α* at the midblastula transition prevents mesodermal gene expression in response to Nodal signals in the vegetal endodermal domain. In support of this idea, we show that Sox17α inhibits endogenous mesodermal gene expression and prevents the induction of mesodermal genes by Nodal signals.

Direct Activation of Sox17α by VegT at the Midblastula Transition

The expression of endogenous *Sox17α* immediately after the midblastula transition and in embryos treated with cycloheximide suggests that *Sox17α* is a direct target of maternal determinants (Yasuo and Lemaire, 1999). In addition, the expression of *Sox17α* in isolated vegetal cells of dissociated embryos suggests that these determinants are not secreted signals, but act cell autonomously (Clements *et al.*, 1999; Yasuo and Lemaire, 1999; Chang and Hemmati-Brivanlou, 2000). Our results demonstrate that the maternal transcription factor VegT is both necessary and sufficient for the initiation of *Sox17α* expression at the midblastula transition and that VegT can activate *Sox17α* in the presence of cycloheximide, supporting a direct role for VegT in initiating *Sox17α* expression. Although the initiation of *Sox17α* expression occurs in the absence of Nodal signaling, *Sox17α* expression becomes dependent on Nodal signals soon after the midblastula transition. The maintenance of *Sox17α* expression by factors other than VegT may be important given the rapid loss of maternal *VegT* transcripts and protein during gastrulation (Stennard *et al.*, 1999). We note that Xanthos *et al.* (2001), using antisense and dominant-negative approaches, have concluded that both the onset and maintenance of *Sox17α* expression is dependent on Nodal function. In this previous study, the dependence of *Sox17α* expression on Nodal function was examined at the gastrula stage (stage 10.5), a point at which Nodal signals are clearly required for maintenance of *Sox17α* expression. However, the requirement for Nodal signals in initiating *Sox17α* expression at the midblastula transition was not examined. In our experiments, the regulation of *Sox17α* by Nodal at the midblastula stage has been examined and we show that VegT initiates *Sox17α* expression independent of Nodal function.

In contrast, *Mxr* expression is initiated later than *Sox17α*, and *Mxr* is not expressed in dissociated embryos, suggesting that *Mxr* is regulated in a noncell autonomous manner (Clements *et al.*, 1999; Yasuo and Lemaire, 1999; Chang and Hemmati-Brivanlou, 2000). Our results show that *Mxr*, like *Sox17α*, is dependent on both VegT and Nodal function at the gastrula stage. However, in contrast to *Sox17α*, VegT activation of *Mxr* is dependent on Nodal signals and *Mxr* is not an immediate-early target of VegT. These data suggest that *Mxr* is regulated by VegT in an indirect, Nodal-dependent manner. Our analysis of *Sox17α* and *Mxr* regulation in the vegetal pole supports the models of endoderm formation proposed by Clements *et al.* (1999) and Yasuo *et al.* (1999).

The importance of TGFβ signals for endogenous endodermal gene expression has been demonstrated using dominant-negative signaling components (Gamer and Wright, 1995; Henry *et al.*, 1996; Chang and Hemmati-Brivanlou, 2000), but the broad specificity of these approaches has limited the ability to assess the role of individual TGFβ family members. Cer-S has been shown in functional assays to inhibit signaling by Xnr1, Xnr2, Xnr4, Xnr5, and Xnr6, but not Activin, Vg1, or Derriere (Piccolo *et al.*, 1999; Agius *et al.*, 2000; Takahashi *et al.*, 2000). Cer-S binds directly to Xnr1 *in vitro*, suggesting that the mechanism of Nodal inhibition by Cer-S is direct (Piccolo *et al.*, 1999). Therefore, our observation that Cer-S inhibits the endogenous expression of *Mxr* and *Sox17α* provides a clear demonstration of the requirement for Nodal signals in *Xenopus* endodermal specification. Consistent with our Cer-S studies, expression of a dominant-negative cleavage mutant of Xnr2 has been shown to reduce the endogenous expression of *Mxr* and *Sox17* (Osada and Wright, 1999), further supporting an essential role for Nodal signals in endodermal gene expression. A similar requirement for Nodal function has been described for endodermal development in the zebrafish. In the zebrafish, genetic studies have demonstrated that the *Nodal*-related genes, *squint* and *cyclops*, are essential for the endogenous expression of

Sox17- and *Mxr*-related genes, and for subsequent development of the endodermal germ layer (Feldman *et al.*, 1998; Rebagliati *et al.*, 1998; Sampath *et al.*, 1998; Alexander and Stainier, 1999; Reiter *et al.*, 2001). Although these studies in *Xenopus* and the zebrafish, including our results with Cer-S, provide compelling evidence for the importance of Nodal signals in endodermal development, these findings do not exclude a role for other TGFβ family members in regulating early endodermal gene expression.

Exclusion of Mesodermal Gene Expression from the Vegetal Endoderm Domain: Sox17α Modifies the Response to Nodal Signals

Nodal signals regulate the formation of endoderm and mesoderm in complimentary, nonoverlapping domains that are defined at the gastrula stage by the vegetal expression of *Sox17α* and the marginal expression of *Xbra*. Our results suggest that the direct activation of *Sox17α* by VegT in vegetal cells may play an important role in spatially limiting the mesodermal response to Nodal signals. The ability of Sox17α to inhibit the marginal zone expression of several mesodermal genes suggests that endogenous Sox17α may prevent mesodermal gene expression in vegetal blastomeres. Consistent with this idea, interference with Sox17 function, using an Engrailed-Sox17β fusion protein, resulted in ectopic expression of *Xbra* in vegetal blastomeres (Hudson *et al.*, 1997). Together, these results suggest that, in addition to promoting endodermal gene expression, Sox17 negatively regulates mesodermal gene expression.

Similar to the effects of Sox17α misexpression in our experiments, Mix.1, Mlk/Bix.2, or Gata5 overexpression also inhibits mesodermal gene expression (Ecochard *et al.*, 1998; Lemaire *et al.*, 1998; Weber *et al.*, 2000). However, in contrast to the endoderm-specific expression of *Sox17α*, the expression of *Mix.1* and *Mlk/Bix.2* extends into the marginal zone and overlaps with mesodermal genes at the early gastrula stage (Ecochard *et al.*, 1998; Lemaire *et al.*, 1998), suggesting that these genes, when expressed at endogenous levels, do not inhibit mesodermal gene expression. Gata5 is expressed in a subset of vegetal cells (sub-blastoporal endoderm) (Weber *et al.*, 2000) and therefore, if Gata5 does prevent mesodermal gene expression in vegetal cells, additional factors would still be required to inhibit mesodermal gene expression in vegetal cells that do not express Gata5. Of the endodermal factors that can inhibit endogenous mesodermal gene expression, only *Sox17* is expressed throughout the vegetal endodermal domain, but not outside of this domain. We note that overexpression of Gata5 has been shown to induce *Sox17α* expression (Weber *et al.*, 2000), suggesting that Gata5 may act through Sox17α to inhibit mesodermal gene expression within the limited *Gata5*-expression domain. It will be interesting to determine whether Mix.1, Mlk/Bix.2, or Gata5 act in parallel to, or upstream of, Sox17α to exclude mesodermal gene expression from the vegetal endodermal region.

Sox17 expression defines the vegetal endodermal domain and Sox17α misexpression is incompatible with the expression of mesodermal genes. These observations raise the possibility that Sox17α modifies the response of vegetal cells to Nodal signals, thus promoting endodermal gene expression and preventing mesodermal gene expression. In support of this idea, we have found that Sox17α expression in animal explants prevents the induction of mesodermal genes by Xnr1, but does not effect the activation of endodermal genes. A number of mechanisms could account for this ability of Sox17α to modify the response to Nodal signals. For example, Sox17α may increase the sensitivity of vegetal cells to Nodal signals. The induction of mesodermal and endodermal genes by Nodal signals is dose-dependent (Jones *et al.*, 1995; Clements *et al.*, 1999; Yasuo and Lemaire, 1999; Agius *et al.*, 2000). Low doses of Nodal induce pan-mesodermal genes (*Xbra*), intermediate doses induce mesodermal and endodermal genes, and high doses induce endodermal genes, but not mesodermal genes such as *Xbra*. Sox17α may cause vegetal cells, or explanted animal cells, to interpret a dose of Nodal that normally induces both endodermal and mesodermal genes, as an effectively higher dose, resulting in the induction of only endodermal genes. However, the high Nodal dose that activates endodermal genes, but not *Xbra*, also induces organizer genes. This model predicts that organizer genes would be expressed throughout the vegetal pole. The absence of vegetal organizer gene expression argues against a potentiation of Nodal activity by Sox17α. Alternatively, Sox17α may directly or indirectly effect the transcriptional competence of mesodermal genes in vegetal cells. In a direct mechanism, Sox17α may prevent transcriptional activation of mesodermal genes by physically interacting with the nuclear Smad2/Smad4 complex downstream of Nodal signals or by binding to distinct elements of mesodermal gene promoters. Given that Sox17α functions as a transcriptional activator, an indirect mechanism seems more likely, with Sox17α activating vegetal expression of a factor that prevents mesodermal gene transcription. Another mechanism is suggested by the ability of Sox17 proteins to inhibit Wnt signaling by direct binding to βcatenin (Zorn *et al.*, 1999). Despite the extensive characterization of Wnt signaling in dorsoventral patterning of the mesoderm, a role for Wnts in the establishment of *Xenopus* mesoderm has not been demonstrated. Therefore, it seems unlikely that an interaction of Sox17 with Wnt signals is responsible for Sox17 inhibition of mesodermal gene expression. Defining the mechanism by which Sox17α modifies the response of vegetal cells to Nodal signals will not only provide insight into germ layer formation, but may also elucidate the mechanisms of Nodal function in other developmental contexts, including left–right patterning and midline development.

The results suggest that one important function of Sox17α is to modify the response of vegetal cells to Nodal signals, but this is unlikely to be the only function of Sox17α. Sox17α can also activate the expression of late endodermal genes, including *Edd*, *Xlhbox8*, and *IFABP*, in animal explants (Hudson *et al.*, 1997). The absence of

endogenous Nodal signals in animal regions (Jones *et al.*, 1995; Agius *et al.*, 2000; Faure *et al.*, 2000) suggests that Sox17α induction of these late endodermal genes in animal cells is independent of Nodal signals. However, we show that Sox17α is insufficient at the gastrula stage to induce the expression of early endodermal markers. Perhaps Sox17α regulates endodermal fate at early stages by inhibiting the mesodermal response to Nodal signals, and at late stages by more directly regulating the expression of endodermal genes. It is also possible that Sox17α upregulates as yet unidentified endodermal genes at the gastrula stage. However, until such Sox17-responsive genes are identified in the gastrula, prevention of a mesodermal response to Nodal signaling seems the most likely mechanism for the observed effects of Sox17α.

Incorporating this role of *Sox17*α into the current model of endoderm formation, we suggest that maternal VegT directly activates *Sox17*α and *Nodal*-related gene expression at the midblastula transition (Fig. 8A). At the late blastula and early gastrula stages (Fig. 8B), Nodal proteins maintain *Sox17*α expression and activate other endodermal genes, including *Mxr*. Due to the early expression of *Sox17*α, mesodermal genes, including *Xbra*, *MyoD* and others, are not activated in vegetal cells by Nodal signals. Furthermore, marginal zone cells, which do not express *Sox17*α, respond to Nodal signals by expressing mesodermal genes. Why marginal cells fail to express *Sox17*α or other endodermal genes in response to Nodal signals awaits further analysis.

Orthologs of the genes regulating *Xenopus* endodermal specification also regulate endoderm formation in the zebrafish embryo. An endoderm-specific *Sox17* ortholog has been isolated (Alexander and Stainier, 1999), and mutations in *Bonnie and Clyde*, a *Mix*-related gene (Kikuchi *et al.*, 2000), *Gata5* (Reiter *et al.*, 2001), and the *Nodal*-related genes, *squint* and *cyclops* (Feldman *et al.*, 1998; Rebagliati *et al.*, 1998; Sampath *et al.*, 1998), demonstrate a requirement for these genes in zebrafish endoderm formation. Although similar genes are involved, endoderm formation in the zebrafish differs in a number of ways compared to *Xenopus*. For example, there is at least one maternal *Nodal* gene in zebrafish (*squint*) and therefore, an upstream VegT-like regulator may not be required for zygotic *Nodal* expression. In addition, the zebrafish ortholog of *VegT* (*spadetail*) is not maternally expressed (Griffin *et al.*, 1998) and appears not to regulate *Nodal* or *Sox17* expression. Furthermore, the prospective endoderm and mesoderm arise from overlapping domains that are indistinguishable by fate mapping of the zebrafish gastrula (Warga and Nusslein-Volhard, 1999), suggesting that the two lineages may be derived from a common precursor, or that cells of each lineage are spatially intermingled. Given this spatial overlap, it seems unlikely that a discrete endodermal domain could be defined by the localization of a maternal VegT-like factor. It may be that a zygotic factor such as Sox17 could divert a subset of mesendodermal precursor cells to the endodermal lineage by preventing mesodermal gene expression. This mechanism or others may be responsible for establishing the spatial organization of endoderm and mesoderm in the zebrafish. Ongoing studies in *Xenopus* and the zebrafish will further define the conserved and species-specific mechanisms controlling endoderm formation in the vertebrate embryo.

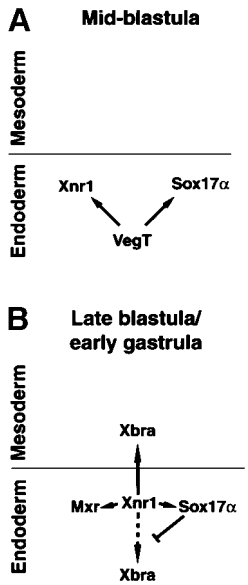

FIG. 8. Direct activation of *Sox17*α by VegT establishes the vegetal endodermal domain by altering the response of vegetal cells to Nodal signals. (A) At the midblastula stage, maternal VegT directly activates *Sox17*α expression independent of Nodal signals. VegT also induces the vegetal expression of *Nodal*-related genes at this stage (Clements *et al.*, 1999; Kofron *et al.*, 1999) (B) At the late blastula and early gastrula stages, Nodal signals maintain the expression of *Sox17*α and induce the expression of other endodermal genes (*Mxr*). The presence of Sox17α in vegetal cells prevents the vegetal induction of mesodermal genes (*Xbra*) in response to Nodal signals. In marginal cells that lack *Sox17*α, Nodal signals induce mesodermal gene expression. This model accounts for the establishment of the vegetal endoderm domain distinct from the mesoderm domain in the early *Xenopus* embryo. In addition, this model provides a mechanism for the involvement of Nodal signals in the establishment of both the endodermal and mesodermal germ layers, tissues that are spatially and functional distinct.

Note added in proof. *casanova*, a zebrafish mutation that results in embryos lacking endoderm, has recently been identified as a Sox-related gene distinct from Sox17 (Dickmeis *et al.*, 2001; Kikuchi *et al.*, 2001). *casanova* loss-of-function results in a failure to express Sox17 and a conversion of endoderm into mesoderm (Dickmeis *et al.*, 2001), consistent with our concusions.

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